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Enzymes and Protein Markers

Evaluation of Nicked Human Chorionic Gonadotropin Content in Clinical Specimens by a Specific Immunometric Assay

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We report the development and characterization of an IRMA for the direct measurement of nicked human chorionic gonadotropin (hCGn) in blood and urine. hCGn derived from a reference preparation of hCG used as an immunogen elicits monoclonal antibodies (mAbs) with enhanced recognition of human luteinizing hormone epitopes. The most specific assay for pregnancy hCGn is an IRMA composed of one mAb to choriocarcinoma-derived hCGn (C5) and a second mAb developed from immunization with normal-pregnancy hCGn. This assay was used to evaluate hCGn profiles in normal, in vitro fertilization, Down syndrome, and ectopic pregnancies. In all pregnancies, hCGn was usually present in much lower concentrations than the non-nicked hCG isoform. Our results suggest that some form of physical separation from the overwhelming quantities of non-nicked hCG present in clinical specimens will be required before accurate immunochemical estimations of hCGn can be made.

Human chorionic gonadotropin (hCG)[5] is a member of the glycoprotein hormone family, which also includes human luteinizing hormone (hLH), human follicle-stimulating hormone, and human thyroid-stimulating hormone. These hormones are heterodimeric, sharing a common alpha subunit but each having a unique beta subunit structure that confers hormone specificity. hCG is produced primarily by the placental trophoblast, serving to maintain steroid production by the corpus luteum in early pregnancy; it is also produced in very small quantities by the pituitary in both men and women *(1–4)*.

Early reports of cleavages (nicks) in the hCG beta subunit at positions 44–45 and/or 47–48 *(5–8)* have been followed by other reports relating to the extent of nicking in various hCG preparations *(9)* and to the biochemical and immunological characterization of nicked hCG (hCGn) *(10)*. Cleavages in the hCG beta sequence have been shown to have profound effects on the biological activity of hCG, reducing the biological activity to <20% of the activity of the parent molecule *(11)*. Nicking also substantially reduces the binding of hCG by many specific monoclonal antibodies (mAbs), some of which are in widespread use as assay reagents *(11, 12)*. Hoermann et al. *(13)* established that nicking eliminates two distinct epitopes present on the intact hCG molecule. These investigations also determined in a study of hCGn in testicular cancer that hCGn was more prevalent in urine than in serum but that its determination did not increase the efficacy over measuring just the intact, non-nicked molecule as a tumor marker.

One consequence of this altered immunological recognition contributes to very extensive discrepancies in hCG values obtained from a variety of commercial hCG kits *(14, 15)*. Although the presence of nicking has been demonstrated clearly by isolation and gel electrophoresis, analysis in biological specimens has generally been indirect, performed either by measuring total intact hCG (i.e., nicked + non-nicked) and subtracting intact non-nicked hCG from the total, the remainder being ascribed to

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[5] Nonstandard abbreviations: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; hCGn, nicked human chorionic gonadotropin; mAb, monoclonal antibody; hCGβn, nicked hCG free beta subunit; hCGβ, hCG free beta subunit; TFA, trifluoroacetic acid; hCGβcf, hCG beta core fragment; BSA, bovine serum albumin; PBS, phosphate-buffered saline; and IVF, in vitro fertilization.

hCGn. Another approach attempted to neutralize ("scavenge") the interfering molecules in assays that did not efficiently discriminate between the nicked and non-nicked form of hCG. However, there are problems, at least in our hands, associated with either of these approaches to assay specificity. We here detail our development of mAbs that distinguish nicked from non-nicked intact hCG, the development of an IRMA for its measurement, and our initial findings related to the assay of hCGn in various forms of clinical pregnancy by direct measurement without prior isolation or pretreatment of the clinical specimens. We also report our findings regarding the use of scavenger antibodies to neutralize the influence of the cross-reacting non-nicked hCG in the clinical specimens under investigation.

Materials and Methods

HORMONES

hCG standard CR 127 was prepared and characterized as described previously *(10, 16)*. Non-nicked hCG (preparation 814) was separated from the parent CR 127 by hydrophobic chromatography and used for cross-reactivity testing and as the hCG assay calibrator. The procedure was a modification of the separation on Phenyl Sepharose described previously *(17)*. CR 127 hCG (26 mg) was dissolved in 0.6 mol/L ammonium sulfate buffer containing 0.05 mol/L ammonium bicarbonate. This solution (3 mL) was loaded on to a Pharmacia Hi Load Phenyl Sepharose prepacked column and eluted by a wash of 90 mL of starting buffer followed by a batch elution with 210 mL of 0.05 mol/L ammonium bicarbonate. During this step of the separation, hCGn eluted along with a small quantity of non-nicked hCG, presumably of a more hydrophilic form than the majority of non-nicked hCG molecules. The major component of the non-nicked hCG was then eluted with 400 mL/L ethanol in 0.05 mol/L ammonium bicarbonate (90 mL).

hCGn (preparation 813) was purified from the parent CR 127 by hydrophobic chromatography as described for non-nicked hCG above, which is a gradient modification of the earlier batchwise separation *(17)*.

hCGn free beta subunit (hCGβn; preparation 834) was separated from CR 129 hCG free beta subunit (hCGβ) by reversed-phase chromatography on a Vydac C4 column with 1 mL/L trifuoroacetic acid (TFA) in distilled water as buffer A and 1 mL/L TFA in acetonitrile as buffer B *(10)*. The semipreparative column size was 22 cm × 10 mm i.d. The flow rate was 2 mL/min, and the gradient was 0–40% buffer B in 60 min.

The hCG beta core fragment (hCGβcf; preparation 455) was prepared from Diosynth crude commercial hCG by modification of the method of Blithe et al. *(18)*, which included gel filtration, Concanavalin A chromatography, and anion-exchange chromatography, followed by reversed-phase chromatography in 1 mL/L TFA in acetonitrile. We added the reversed-phase step, which improves the amino acid analysis of the hCGβcf.

The hLH (AFP-8270B) and the hLH free beta subunit (AFP-3282) were kindly provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. The hLH beta core fragment was prepared as described by Birken et al. *(19)*.

PURIFICATION OF mAbs

Immunoglobulins were purified from ascites by the Protein A Monoclonal Antibody Purification System (Bio-Rad). The protein concentration of pure antibodies was determined by amino acid analysis. Purification of mAbs was checked by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate according to the method of Laemmli *(20)*.

IODINATION OF HORMONES AND mAbs

Antibodies and hormones were labeled with ^{125}I by the chloramine T method *(21)*.

IMMUNIZATION OF MICE AND CELL FUSION

Balb/c mice were immunized with hCGn (preparation 813) according the following protocol: the first immunization was performed by the subcutaneous injection of 15–20 μg of immunogen per animal in complete Freund's adjuvant; the second immunization was performed 2 weeks later by injection of the same amount of hormone in incomplete Freund's adjuvant. On days 21 and 28, the mice received intraperitoneal immunizations, using 15 μg of antigen in phosphate-buffered saline (PBS) for each animal. Immune sera were tested in a liquid phase RIA using [^{125}I]hCGn. Mice with a high immune response were boosted with 15 μg of hormone and, after 3 days, used for fusion.

Splenocytes from immunized mice were fused with cells of myeloma line X63-Ag8.653 (American Type Culture Collection) 3 days after the booster injection, according to the method of Kohler and Milstein *(22)* as described by Kovalevskaya et al. *(23)*. The splenocyte:myeloma cell ratio was 4:1 or 6:1. Polyethylene glycol 4000 (Sigma Chemical Co.) was used as the fusing reagent. After fusion, cells were distributed in six microtitration plates that contained mouse peritoneal feeder cells and cultured for 1 week in hypoxanthine-aminopterin-thymidine-RPMI 1640 containing 200 mL/L fetal calf serum (HyClone). One-half of the medium was replaced every 3 days. On days 12–14 post fusion, culture supernatants (100 μL) from the wells with cell clones were screened for the presence of antibodies to hCGn, using a liquid phase RIA (vide infra). Positive selected cells were cloned at least two times by the limiting-dilutions method on mouse peritoneal feeder cells. Balb/c mice received intraperitoneal injections of the subclones (0.5×10^6 to $\times 10^6$ cells/mouse), and mAbs were purified from the ascites as describe previously.

Isotypes of mAbs were determined using the Immuno-Pure Monoclonal Antibody Isotyping Kit II (AP/PNPP;

Pierce) according to the manufacturer's instructions for the antigen-dependent technique.

SCREENING OF PRIMARY CLONES AND IMMUNE SERUM

Primary screening was carried out in a liquid phase RIA with [^{125}I]hCGn, using a procedure described previously (*24*). Briefly, the binding buffer consisted of PBS supplemented with 1 g/L bovine serum albumin (BSA) and 1 g/L sodium azide. One hundred and fifty microliters of solution (containing 30 000–40 000 cpm [^{125}I]hCGn) was added to 100 μL of culture supernatant diluted 2.5:1 with PBS. Fifty microliters of 80 mL/L normal mouse serum was also added. This solution was incubated first for 1 h at 37 °C and then overnight at 4 °C. The next day, 500 μL of a 25 mL/L goat antimouse serum was added, and the mixture was incubated for 1 h at 37 °C, followed by incubation for 2 h at room temperature. The precipitate containing bound radioactive hormone was separated by centrifugation and counted in a gamma counter. Supernatants of positive clones were tested in the same type of assay to check preliminary cross-reactivity with [^{125}I]hCG and [^{125}I]hCGβ. Immune serum was used as a positive control.

COMPETITIVE LIQUID PHASE RIA

Our competitive liquid phase RIAs have been described previously (*23*). Briefly, cell supernatants were used at those dilutions at which ~40% of maximum antibody binding occurred in the absence of unlabeled hormone. The following reagents were added to each 12 × 75 mm polystyrene tube: 100 μL of diluted supernatant, 30 000–40 000 cpm of [^{125}I]hCGn in 300 μL of binding buffer (PBS, pH 7.2, containing 1 g/L BSA, and 1 g/L sodium azide), 100 μL of competitor solution, and 100 μL of 80 mL/L normal mouse serum. After incubation for 1 h at 37 °C and overnight at 4 °C, 1 mL of 25 mL/L goat antimouse serum was added as in the primary screening. Affinity constants were calculated by homologous competitive displacement assays using the personal computer version of the program Ligand by Munson and Rodbard (*25*).[6] In our RIA format, cross-reactivity was calculated as a percentage of the molar ratio of hCGn to competitor at 50% of maximum binding.

IRMA FOR hCGn

Our methodology for the construction and validation of IRMAs has been described fully (*26*). Briefly, the specificity of the antibody pairs and their capacity for simultaneous binding to antigen were determined as follows.

The capture antibody was adsorbed onto the wells of microtiter plates (Immulon IV; Dynatech) by incubating a 10 mg/L solution in coating buffer (0.2 mol/L bicarbonate, pH 9.5) overnight at 4 °C. The coating antibody solution was aspirated, and the plates were washed (wash solution: 9 g/L NaCl, 0.5 g/L Tween 20) and blocked with a 10 g/L solution of BSA in PBS containing 1 g/L sodium azide. After incubation with the BSA solution (minimum of 3 h at room temperature), the blocking solution was removed, the wells were washed again with wash solution, and 200 μL/well of the appropriate hCGn calibrators or potential cross-reacting molecules was added in phosphate buffer B (PBS containing 1 g/L bovine gamma globulin and 1 g/L sodium azide) or in hCG-free serum (for serum assay; Chemicon). After overnight incubation at 4 °C, the plates were again aspirated and washed, and 200 μL (50 000–100 000 cpm) of ^{125}I-labeled antibody was added to the wells, which were again incubated for 24 h at 4 °C. The tracer was aspirated, the plates were washed with wash solution, the individual wells were placed in glass tubes, and the radioactivity was determined in a Packard Cobra gamma counter. Doses were determined by interpolation from a smoothed spline transformation of the data points.

The analytes tested for potential cross-reaction with the normal-pregnancy hCGn mAbs included hCG, hCGβ, hCGβn, hCGβcf, hLH, the hLH free beta subunit, and the hLH beta core fragment. In the IRMA format, cross-reactivity was calculated as a percentage of the molar ratio of hCGn to tested analyte at 50% of maximum binding.

In addition to the hCGn assay, the B109-B108 IRMA for intact hCG was used (*26*).

RECOVERY OF hCGn

hCGn was added to hCG-free human serum and urine. hCGn was also assayed as described above, using the B151-B604 assay in buffer B in the presence of increasing concentrations of hCG (1.76–176 nmol/L) with or without the addition of 10 mg/L B109 as a scavenger for nonnicked hCG.

SUBJECTS

Down syndrome samples (n = 9) and control normal-pregnancy urine samples (n = 99), in vitro fertilization (IVF) normal-pregnancy samples (n = 42), IVF ectopic pregnancy samples (n = 9), and spontaneous abortion samples (n = 12) were the kind gift of Dr. L. Cole (Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT). Nine matched serum/urine samples from the first trimester and five urine samples from the third trimester were provided by Dr. A. Kelly at Columbia Presbyterian Medical Center, New York, NY.

Trophoblast disease serum (17 samples) and urine (28 samples) were also obtained from Dr. Cole, but were collected by Dr. Edward Newlands (Charing Cross Hospital, London, UK).

All samples were stored frozen at −20 °C until they were assayed. Because extreme values of sample pH may

[6] IBM personal computer version of program distributed by P. Munson, Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, Bethesda, MD 20892 in 1993.

interfere with antibody binding, the urine pH was adjusted with 1.0 mol/L Tris (pH 9.0, 50 mL/L of urine) before being assayed, so that the final pH was 7–7.4.

CREATININE

Creatinine determinations were performed in a 96-well microtiter plate format by a procedure adapted from Taussky (*27*).

STATISTICAL ANALYSIS OF DOWN SYNDROME VS NORMAL-PREGNANCY URINE

Normal-pregnancy urine samples (n = 99) and Down syndrome samples (n = 9) were tested for intact hCG in the B109-B108 assay and for hCGn in the B151-B604 assay. Data were normalized to creatinine. To minimize the effects of outliers on the analysis any values greater or less than 2 SD from the mean were excluded from the analysis. Even after removal of these extreme values, the data remained highly skewed, and these remaining values were then log-transformed to approximate gaussian distributions. The data were then analyzed by a Generalized Additive Model approach (*28*).

Results and Discussion

CHARACTERIZATION OF REAGENTS USED

Preparations of all hormones were characterized by amino acid sequence analysis and amino acid compositional analysis. In addition, the nicked and non-nicked hCG preparations were characterized by reversed-phase HPLC in the TFA–acetonitrile systems (see *Materials and Methods*). These systems were used to estimate the quantity of hCGβn compared with hCGβ in each preparation. The non-nicked hCG displayed virtually no hCGβn, as shown by its HPLC profile in Fig. 1. Amino acid sequence analysis detected no peptide bond nicks. The most sensitive method for detecting peptide bond nicking involves reduced sodium dodecyl sulfate gel electrophoresis followed by blotting and staining of the blot with antibodies to the hCGβ COOH-terminal region (*17*). Such blots of hCGn or hCGβn indicate two beta bands, one representing $Beta_{48-145}$ and the other representing $Beta_{1-145}$. Blots indicated that some hCGβn remains in this preparation, but the quantity is likely ≤1%, based on the HPLC profile. The hCGn preparation clearly contains non-nicked hCGβ (Fig. 1). The integrated value of the hCGβ peak was compared with the total peaks of the alpha and beta subunits (nicked and non-nicked); it was then calculated that this preparation contained ~20% non-nicked hCGβ.

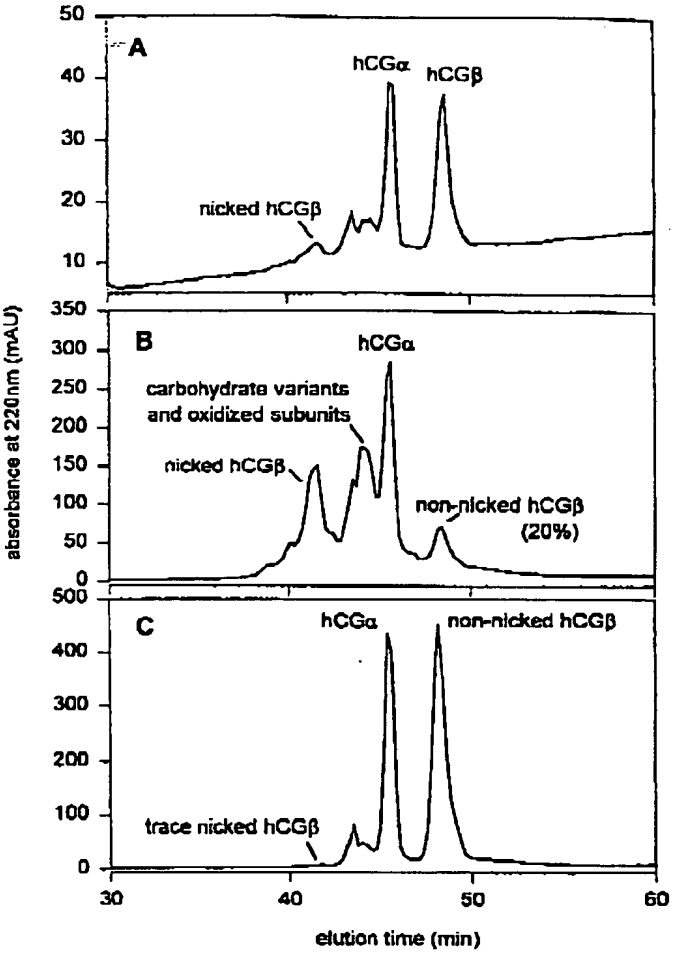

Fig. 1. Chromatographic characteristics of the forms of hCGn (*B*) and non-nicked hCG (*C*) separated from parent CR 127 hCG (*A*).

Reversed-phase HPLC separations in TFA–acetonitrile systems, using a C4 Vydac column. *A*, parent CR 127 hCG preparation separated by hydrophobic chromatography into a nicked-enriched form of hCG (prep 813; *B*) and a nick-reduced preparation of hCG (prep 814; *C*).

mAb DEVELOPMENT AND CHARACTERIZATION

The development of mAbs to hCGn is challenging because of the extensive structural homology between intact hCG and its nicked forms. As immunogen, we used hCGn purified from a urine pool collected from an hCG reference preparation (see *Materials and Methods*). Six mAbs to hCGn (B601, B603, B604, B605, B607, and B610) were selected, and each was characterized on the basis of its affinity and specificity. Affinity constants were calculated as 5.3×10^8 L/mol for B601, 0.89×10^7 L/mol for B603, 1.2×10^9 L/mol for B604, 1.4×10^9 L/mol for B605, 1.4×10^7 L/mol for B607, and 4.6×10^7 L/mol for B610. All mAbs were IgG1, κ isotype. Antibodies B601 and B605 recognized hCG and hCGn as well as hLH but did not recognize hCGβ, hCGβn, or hCGβcf. Antibodies B604 and B610 bound to a common epitope present on all hormones evaluated and could be used as universal second antibodies in two-site assays for hCG/hLH. B603 and B607 preferred hCGn to intact hCG (~17% cross-reactivity with hCG in our RIA), but also bind hLH as well as hCGn. In addition, antibody B151 was also evaluated in the same liquid phase RIA format for cross-reactivity with the normal-pregnancy hCGn. Although B151 was developed to the choriocarcinoma hCGn (C5) (*29*) and demonstrated

a slightly higher affinity for C5 hCGn than for the normal-pregnancy hCGn in our RIA assay format (Birken et al., manuscript in preparation), this antibody provided the best discrimination between pregnancy hCGn and intact hCG in our RIA format (4–6% cross-reactivity with hCG and hCGβn; B151 affinity constant for normal-pregnancy hCGn was calculated as 8.0×10^8 L/mol). It has an additional advantage over hCGn mAbs developed to normal-pregnancy hCGn, in that it does not cross-react with hLH. Fig. 2 illustrates mAbs B151 and B604 binding with hCGn and related molecules in a competitive liquid phase RIA where [^{125}I]hCGn was used as a tracer. These two antibodies were determined to be the most specific combination for hCGn detection in the IRMA format.

Data obtained from the liquid phase competitive RIA suggest that nicking of normal-pregnancy hCG alters its structure so that epitopes common to intact hLH are exposed; however, this is not true for B151, which was developed to choriocarcinoma hCGn.

ASSAY CHARACTERIZATION

All antibodies, including B151, were checked in all possible combinations that could provide the basis for construction of two-site IRMAs. The best specificity for the normal-pregnancy hCGn was demonstrated by an assay that used B151 as the capture antibody and B604 for

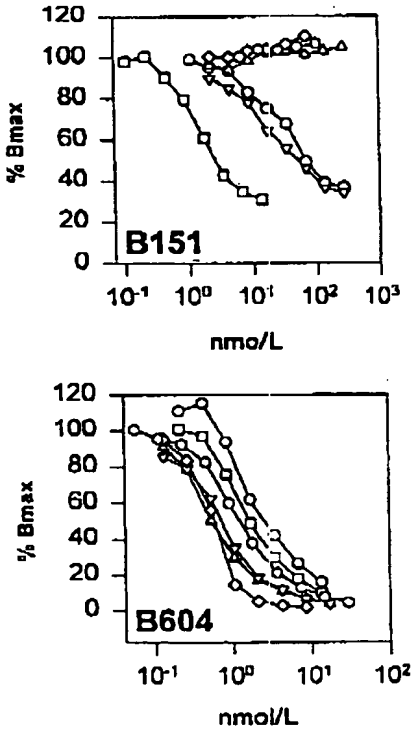

Fig. 2. Binding characteristics of mAbs to normal-pregnancy hCGn in liquid phase competitive assays.

Normal-pregnancy hCGn iodinated with ^{125}I was used as a tracer. O, hCG; □, hCGn; △, hCGβ; ▽, hCGβn; ◇, hCGβcf; · · , hLH.

detection. The detection limit (least detectable dose, which is defined as that concentration of hCGn corresponding to the zero calibrator plus 2 SD) was <2 pmol/L. This assay was characterized by a wide dynamic range (2–4000 pmol/L can be measured without the need for dilution). The measured B151-B604 IRMA cross-reactivity with normal-pregnancy hCG was 2.5%. Because hCGn is contaminated with 20% non-nicked hCG, the true cross-reactivity probably lies close to 2%. The cross-reactivity with hCGβn was 3.7% in the B151-B604 assay. For all other checked analytes the cross-reactivity was <0.1%.

For hCG and hCGn, the intraassay coefficients of variation (CVs) were 5% and 12% for serum, and 6% and 7% for urine, respectively. The interassay CVs were 11% and 13% for serum, and 10% and 14% for urine, respectively.

RECOVERY STUDIES

hCGn recovery from hCG-free serum to which hCGn had been added varied between 69% and 83%; hCGn from hCG-free urine to which hCGn was added was 72%. The recovery in buffer was 78–94%, depending on the hCGn concentration. The lower recoveries obtained in serum and urine compared with buffer may reflect a matrix effect. The common approach to diminishing this effect is sample dilution, which is not an alternative available in this assay because of the low quantities of hCGn present in specimens. As a consequence of the relatively low hCGn concentrations compared with the non-nicked hCG concentrations we observed in clinical specimens, determination of the analytical recovery of hCGn in the presence of the non-nicked variant was an essential component of assay validation. This experiment illustrated that as the concentration of intact hCG in the sample increased, the apparent concentrations of hCGn change dramatically (Fig. 3A). In the case of pregnancy or choriocarcinoma samples with very high concentrations of hCG, the 2–3% cross-reactivity of intact hCG in the nicked assay can appear as a substantial amount of hCGn because of the cross-reactivity with non-nicked hCG in the hCGn assay.

When the cross-reactivity factor is taken into account, it is possible to obtain reasonably accurate measurements of hCGn only over a limited range of values for the both hCGn and non-nicked hCG (Fig. 3A). For example, if hCGn is in the 300–400 pmol/L range and the corresponding values for non-nicked hCG do not exceed 1760 pmol/L, then a reasonably accurate determination of hCGn can be made. If non-nicked hCG concentrations are in the range of 17 600 pmol/L, however, then the hCGn concentration needs to be >800 pmol/L to be measurable. At non-nicked hCG >17 600 pmol/L, the interference from the non-nicked hCG in the hCGn assay becomes too large to provide a useful estimation. Unfortunately, in our examination of clinical specimens thus far, values of

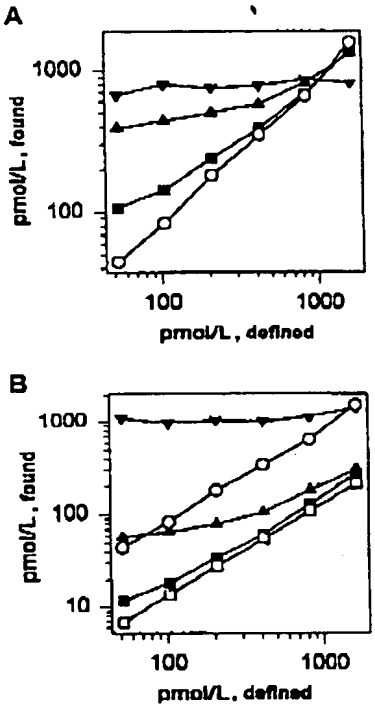

Fig. 3. Recovery of hCGn in B151-B604 assay in the presence of increasing concentrations of hCG (*A*) and in the presence of B109 (0.01 g/L) scavenger for hCG (*B*).

○, hCGn; ■, hCGn + 1.76 nmol/L hCG; ▲, hCGn + 17.6 nmol/L hCG; ▼, hCGn + 176 nmol/L hCG; □, hCGn with 0.01 g/L B109 added. In *B*, all samples with added hCG (■, ▲, ▼) also have 0.01 g/L B109 added.

hCGn have been much below those required for their accurate estimation by our assay.

An approach to this problem has been the use of scavengers to complex the interfering analyte and neutralize its immunological activity (*30, 31*). Our data illustrate that the scavenger itself, unless absolutely specific, can bind hCGn and hence change its apparent concentration (Fig. 3B). This observation is also supported by a slightly different assay format, i.e., B108 was the detection antibody. The influence of different concentrations of B109 scavenger antibody on apparent concentrations of hCGn in this type of hCGn assay is shown on Fig. 4B. The scavenger application decreases the recovered amount of hCGn. Conversely, the scavenger does not totally eliminate the influence of hCG on this assay (Fig. 4A).

When B109 is used as a scavenger for hCG in samples that contain both hCGn and hCG (as occurs in clinical specimens), the resulting equilibrium is complex. The presence of one immobilized mAb on the plate (B151) and the second mAb (B109) together with both hCG and hCGn in solution at the same time produced ambiguous results (Fig. 3). Because neither antibody is absolutely specific for either analyte, both nicked and non-nicked hCG are in binding equilibrium with both the immobilized antibody and the liquid phase scavenger antibody, although with very different affinities. Consequently, the values obtained from this assay format are not reliable. The final results are shown on Fig. 3B. The degree of interference also depends on the concentration of these analytes.

EVALUATION OF hCGn AND INTACT hCG IN SERUM AND URINE IN VARIOUS FORMS OF CLINICAL PREGNANCY

The hCGn content of clinical samples was evaluated with the B151-B604 hCGn-specific assay. It should be kept in mind that these results are reliable only within the relative ranges of hCGn and non-nicked hCG within which cross-reactivity is not a problem. Values in this range were never attained in any of these clinical specimens, however. Thus, when interpreting the values, one must keep in mind that they reflect extremely small amounts of hCGn compared with the much larger quantities of non-nicked hCG present and cannot be construed as representing absolute quantities. In none of the clinical specimens we examined did we discover the quantities of hCGn found by other investigators using indirect assay methodologies.

Urinary hCG and hCGn throughout the normal pregnancy. The median concentration and range of non-nicked hCG and hCGn throughout normal pregnancy is illustrated in Table 1. Both hormones have been normalized to urinary

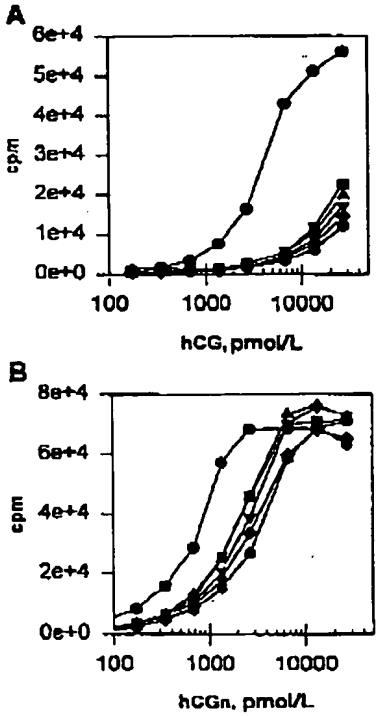

Fig. 4. Effects of different concentrations of B109 (as a scavenger for hCG) on hCG binding (*A*) and on hCGn binding (*B*) in the B151-B108 assay.

B109 concentrations: ●, 0 mg/L; ■, 6.25 mg/L; ▲, 12.5 mg/L; ▼, 25 mg/L; ◆, 50 mg/L; ●, 100 mg/L.

Table 1. Median and range of values for non-nicked hCG and apparent values for hCGn as measured by B151-B604 assay in normal-pregnancy urine.

	Gestational age, weeks	No. of samples analyzed	No. of samples positive in hCGn assay	hCGn (B151-B604) hCGn, pmol/g Crt[b] Median	hCGn (B151-B604) hCGn, pmol/g Crt[b] Range	Non-nicked hCG[a] hCG, nmol/g Crt Median	Non-nicked hCG[a] hCG, nmol/g Crt Range
Urine	5–6	9	3	140	111–206	6.1	1.6–31.2
	10.6–14.9	17	17	971	36–6065	214.0	64.8–7184.5
	15–21.7	81	81	113	6–4718	60.5	7.3–2663.5
	28.1–39	6	6	46	28–93	30.2	16.5–138.1
Serum	5–6	9	7	203[c]	10–518[c]	5.9[c]	0.7–21.4[c]

[a] Note that non-nicked hCG is expressed in nmol/g creatinine and hCGn is expressed in pmol/g creatinine.
[b] Crt, creatinine.
[c] Concentration in serum expressed in pmol/L for hCGn and in nmol/L for hCG.

creatinine. It is striking to note that difference between the values for non-nicked hCG and hCGn is greater than three orders of magnitude. This wide difference in concentration persists throughout pregnancy. Previous reports have indicated that the extent of nicking both varied widely in individual pregnancies *(9)* and generally increased as the pregnancies progressed *(32)*.

We did not observe this increase. Indeed, the ratio of the median concentrations of hCG to the hCGn in urine actually increased from 44 in the first trimester to >650 in the third trimester, suggesting that the fraction of hCGn actually declined as the pregnancies progressed (Table 1). The interpretation of these results is clouded, however, because their accuracy is compromised by the interference of the large excess of non-nicked hCG. Because overall hCG concentrations are lower in the third trimester than in the first, the effect we are observing may be a result of the diminished effect of non-nicked hCG in the assay.

Urinary hCG and hCGn in early normal pregnancy, ectopic pregnancy, and spontaneous abortion from IVF patient samples. Table 2 illustrates the median range of values, normalized to creatinine, for the above listed pregnancy types at 2–4 weeks after embryo transfer. In all types of IVF pregnancies, hCGn was either nondetectable or present at such low concentrations that cross-reaction cannot be ruled out as its source.

Trophoblast disease serum and urine. We examined 17 serum and 28 urine samples collected posttreatment from patients with choriocarcinoma to determine whether hCGn, which has been reported to be more prevalent in this condition *(33)*, would be a more sensitive marker for disease recurrence. Because of limited amounts of sample, all of these specimens were run at a 1:10 initial dilution. This increased the limit of detectability to 7 pmol/L in serum and 8 pmol/L in urine (the least detectable doses for these assays were 0.7 and 0.8 pmol/L, respectively). There were only small amounts of non-nicked hCG detected in the sera; hCGn was not detectable in any of these specimens. In the urine, only 2 of 28 specimens had substantial concentrations of hCG (587 and 18 715 pmol/L). Only the latter positive specimen contained a detectable amount of hCGn, again at a concentration (150 pmol/L) that could not preclude cross-reaction as the source.

Matched blood and urine specimens in early clinically normal pregnancy. Nine matched blood and urine specimens collected at 5–6 weeks gestational age were evaluated for hCGn content (data incorporated in Table 1). In the blood, seven of nine samples had detectable hCGn, ranging from 0.1% to 4.6% of the corresponding value for intact, non-nicked hCG. However, although high values of hCG were associated with increased values of hCGn, there was no significant correlation between the two numbers. In the corresponding urine samples, there were only three samples positive for hCGn; all three corresponded to specimens in which the highest values for non-nicked hCG were present.

Table 2. Median and range of values for urine non-nicked hCG and apparent values for hCGn as measured by B151–B604 assay in IVF patients.

Diagnosis	Gestational age, weeks	No. of samples analyzed	hCGn (B151–B604) No. of samples positive in hCGn assay	hCGn (B151–B604) hCGn, pmol/g Crt[b] Median	hCGn (B151–B604) hCGn, pmol/g Crt[b] Range	Non-nicked hCG[a] hCG, pmol/g Crt Median	Non-nicked hCG[a] hCG, pmol/g Crt Range
Normal pregnancy	1.7–4.0	42	15	23	3–222	2.7	0.2–88.5
Ectopic pregnancy	2.3–4.3	9	4	6	3–20	3.6	1.2–6.7
Spontaneous abortion	1.9–4.1	12	3	4.5	4–11	0.8	0.2–2.3

[a] Note that non-nicked hCG is expressed in nmol/g creatinine and hCGn is expressed in pmol/g creatinine.
[b] Crt, creatinine.

Down syndrome and control normal urine for the same gestational age. Nine urine samples from Down syndrome patients were tested in B109-B108 and B151-B604 assays and compared with 99 normal-pregnancy samples matched for gestational age (Table 3 and Fig. 5). Statistical analysis revealed that the distributions of hCG and hCGn were highly skewed, even after extreme values were excluded. To improve the distribution, log-transformation was used. There was significant correlation between the gestational age (in weeks) and log(hCG) and log(hCGn) among non-Down syndrome subjects ($r = -0.28$, $P = 0.006$; and $r = -0.39$, $P = 0.0001$, respectively) and a correlation of a similar magnitude but lacking significance among Down syndrome subjects ($r = -0.39$, $P = 0.34$; and $r = -0.23$, $P = 0.55$, respectively); this is mainly attributable to the small sample size in the Down syndrome group. The Fisher z-test revealed no significant difference in the gestational age (in weeks) between the two groups: for log(hCG), $z = -0.17$, $P = 0.865$; and for log(hCGn), $z = 0.25$, $P = 0.802$. To examine the difference of log(hCG) and log(hCGn) between the two experimental groups, we used a generalized additive model, which permits adjustment for the effect of gestational age through a nonlinear smoothing function. Although the concentrations of both hCG ($P = 0.0057$) and hCGn ($P = 0.0009$) were significantly higher in the Down syndrome group than in the normal-pregnancy group, it is impossible to state with certainty that this change in hCGn represents a true increase or is just a reflection of the increased amounts of non-nicked hCG.

Because hCGn was expressed at such low concentrations relative to the intact molecule in other forms of pregnancy, extensive statistical analysis on pregnancy categories other than Down syndrome was not performed.

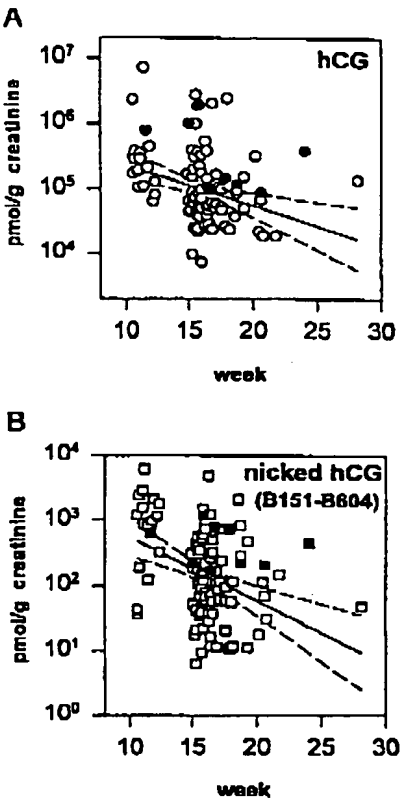

Fig. 5. hCG as measured by the B109-B108 assay (*A*) and hCGn as measured by the B151-B604 assay (*B*) in urinary Down syndrome (n = 9) and normal-pregnancy (n = 99) samples plotted as a function of gestational age.

Open symbols, normal-pregnancy samples; *filled symbols*, Down syndrome samples. Regression curves and 95% confidence intervals are shown for normal-pregnancy samples.

Conclusions

When hCGn purified from a normal-pregnancy pool is used, it is possible to develop mAbs with an acceptable specificity, especially considering the nearly identical structural homology between the nicked and non-nicked forms of hCG and the difficulty in preparing perfectly pure nicked and non-nicked hCG calibrators.

Nicking of normal-pregnancy hCG appears to expose hCG epitopes common to hLH. This effect was not observed in mAb B151 raised against choriocarcinoma-derived hCG (C5), which was 100% nicked.

It appears that only small quantities of hCGn are present in most clinical specimens. An assay of virtually perfect specificity is therefore required to accurately measure these concentrations. However, the use of scavenger antibodies to increase the specificity of our assay, although somewhat effective, also introduced other interferences into the measurement, again producing compromised results.

Table 3. Median and range of values for urine non-nicked hCG and apparent values for hCGn, as measured by B151–B604 assay in Down syndrome vs normal-pregnancy patients.

			hCGn (B151–B604)		Non-nicked hCG[a]	
			hCGn, pmol/g Crt[b]		hCG, nmol/g Crt	
Diagnosis	Gestational age, weeks	No. of samples analyzed	Median	Range	Median	Range
Normal pregnancy	10.6–28.1	99	131	6–6065	74	7.25–7184.5
Down syndrome	11.6–24	9	459	176–1168	148.4	86.8–1870.5

[a] Note that non-nicked hCG is expressed in nmol/g creatinine and hCGn is expressed in pmol/g creatinine.
[b] Crt, creatinine.

An alternative methodology, that of making a measurement with an assay that is indifferent to nicking ("total hCG"), subtracting from it the hCG concentration found in an assay which does not measure hCGn ("non-nicked hCG"), and assigning the value of the difference to hCGn is risky in the absence of an independent criterion for ascertaining the accuracy of the initial assumption.

It is well-documented that hCG exists in multiple isoforms that change throughout pregnancy. For the subtractive approach to be valid, the two assays chosen must have the same measuring characteristics vis a vis all of these isoforms except hCGn, a requirement that may not always be fulfilled and is extremely difficult, if not impossible, to demonstrate. We illustrated this observation recently in a study of hCG expression in early pregnancy and early pregnancy loss, where we showed that the difference between total hCG and non-nicked hCG does not represent hCGn but rather multiple hCG isoforms recognized differentially, at least in early pregnancy specimens, by the two hCG assays we used (*34*).

The main finding with respect to the application of our hCGn specific assay is that the values for hCGn in clinical specimens were too small to be measured reliably in the presence of large quantities of non-nicked hCG. We clearly failed to detect hCGn in the quantities described by other investigators using indirect assay techniques (*12, 14*). Our results, with an assay that probably has the best specificity practically achievable, remain unsatisfactory. Our findings clearly show that some additional separation technique must be applied to the clinical specimens before they can be assayed in our system. We have shown that an in situ separation, as exemplified by the scavenger approach, is not satisfactory. Unfortunately, the partial purification afforded by chromatography is not applicable to the analysis of anything but small numbers of samples.

Our results present a conundrum. The existence of hCGn has been established by several investigators, including ourselves, using isolation of hCGn from urine and sequence analysis (*9, 10*). Having unequivocally established its existence, however, we do not know its origin and are unable to measure it with our assay, except rarely in some patients with a malignancy in which there is no overwhelming amount of non-nicked hCG present to interfere.

However, the hCGn preparations that were characterized by primary structural analysis were all from nonsterile urine collections. It is possible that microbial growth in the urine produced additional protease activity. In fact, a recent report demonstrates that microbial action in serum is the origin of hCGn and hCGβn (*30*). Microbial proteases are also a likely source of nicking in the reference hCG preparations, such as CR 127, that are derived from Diosynth crude commercial hCG (*10*). The peptide bond cleavage found at β_{44-45} is not usually found in urine isolated from individual healthy pregnant women (*10–12, 29*). This implies a bacterially generated protease activity (*10–12*). It is known that loop 2 of hCGβ is highly sensitive to peptide cleavage because it is completely exposed to solvent (*35*) and is easily cleaved by trypsin (*36*) or elastase (*10*).

Another possibility might be that the assay for hCGn is subject to some matrix interference present in serum or urine. However, our recovery studies of hCGn added to serum or urine, although not quantitative, nevertheless demonstrate that authentic hCGn added to either serum or urine, is recoverable in our assay. In summary, using our newly developed direct immunometric assay for hCGn, we cannot confirm previously published reports of significant quantities of hCGn produced during normal pregnancies. We must, therefore, raise the question of whether the observed presence of peptide bond cleavages in the highly protease-sensitive loop 2 region are caused by microbial growth during the collection and processing of urine.

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